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14. ABSTRACT

Several groups have demonstrated that women with BRCA1 germline mutations are more likely to have breast cancers that are basal-like by gene expression profiling. While BRCA1 germline mutations are uncommon, and contribute to fewer than 5% of breast cancer cases, our lab has demonstrated that methylation of the BRCA1 promoter occurs to some degree in almost 50% of high-grade, hormone receptor negative sporadic tumors. As promoter methylation leads to transcriptional repression, we propose that such tumors will be sensitive to DNA damaging agents and resistant to microtubule inhibitors, given the role that BRCA1 plays in both DNA repair and cell cycle. My laboratory has generated preliminary in vitro data that demonstrates breast cancer cells with BRCA1 promoter methylation and low expression levels are three-fold more sensitive to cisplatin and ten times more resistant to paclitaxel, as compared to cells with normal BRCA1 expression.

15. SUBJECT TERMS
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INTRODUCTION

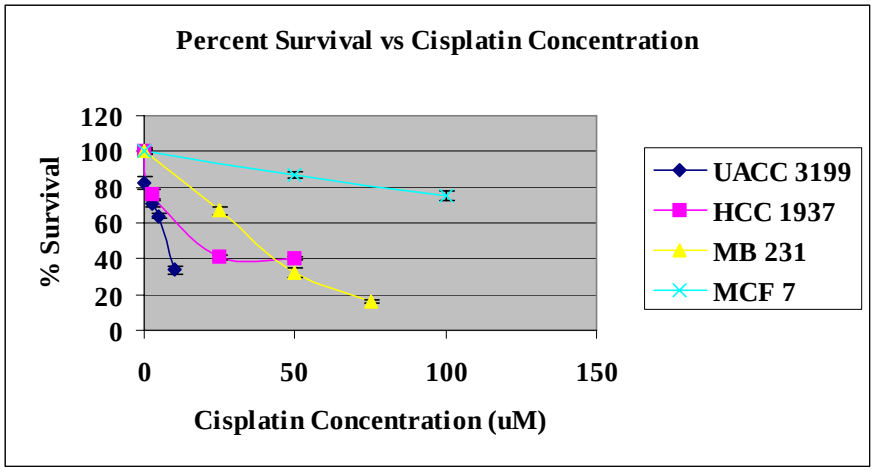
Several groups have demonstrated that women with *BRCA1* germline mutations are more likely to have breast cancers that are basal-like by gene expression profiling[1, 2]. While *BRCA1* germline mutations are uncommon, and contribute to fewer than 5% of breast cancer cases, epigenetic alterations in *BRCA1* occur with much greater frequency. Our lab has previously demonstrated that heterogenous methylation of the *BRCA1* promoter occurs in almost 50% of high-grade, hormone receptor negative sporadic tumors[3]. Given the role of *BRCA1* in both DNA repair and cell cycle regulation, it is likely that cells deficient in BRCA1 secondary to methylation will be sensitive to DNA damaging agents and resistant to microtubule inhibitors, as has previously been demonstrated for cells deficient in BRCA1 secondary to mutation. The role of *BRCA1* methylation in determining chemosensitivity is unknown.

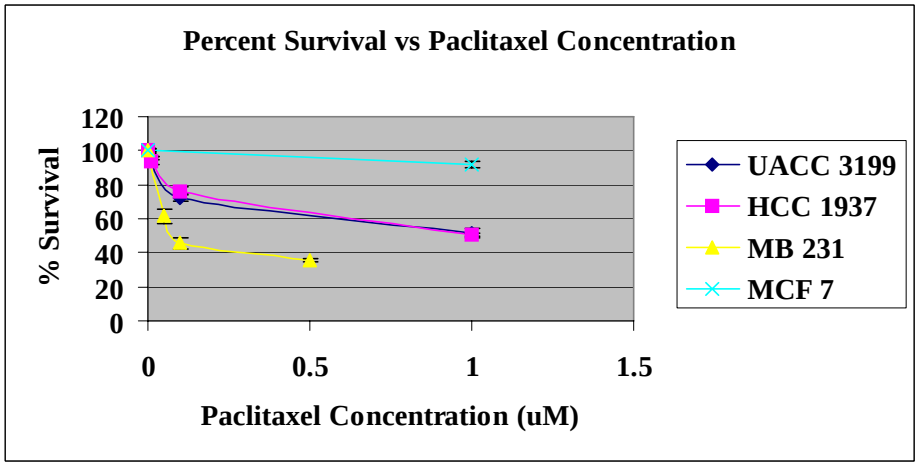
BODY

Task 1: To test *BRCA1* normal, *BRCA1* mutated and *BRCA1* methylated breast cancer cell lines in vitro for sensitivity to chemotherapeutic agents commonly employed in breast cancer treatment.

Several groups have demonstrated that cells deficient in BRCA1 secondary to mutation are sensitive to cisplatin and resistant to paclitaxel, as compared to BRCA1 competent cells[4, 5]. We hypothesized that cells deficient in BRCA1 secondary to promoter methylation would also be sensitive to cisplatin and resistant to paclitaxel. To test this hypothesis, we developed an *in vitro* model consisting of four breast cancer cell lines. UACC-3199 is a breast cancer cell line that is almost completely methylated at all 30 CpG sites located at the 5' end of the *BRCA1* gene. UACC-3199 has no BRCA1 protein expression secondary to promoter hypermethylation[6]. HCC-1937 is a breast cancer cell line derived from a patient with a germline mutation in *BRCA1*. This cell line has one mutant allele that produces a truncated form of the BRCA1 protein[7]. Two well characterized breast cancer cell lines MCF-7 (ER positive, *BRCA1* normal) and MDA-MB-231 (ER negative, *BRCA1* normal) have been used to provide the context for determining relative sensitivity of the *BRCA1* methylated and mutated cells lines as compared to those with normal *BRCA1* expression.

To evaluate cell survival after drug exposure, exponentially growing cells were exposed to drug at escalating dose levels in triplicate. Untreated cells served as a control. Cells were exposed to drug for 24 hours. Cells were harvested 96 hours after exposure and were stained with Annexin-V-FITC and DAPI. Cell survival and apoptosis were determined by flow cytometry using FACS DiVa. FACS Flowjo analysis software (version 6.1.1) was used to generate percent apoptotic and live cells. Cells that were positive for Annexin and negative for DAPI were considered apoptotic, cells that were double negative were considered live, and cells that were double positive were considered dead. Each experiment was repeated three times. Each experiment was normalized to its own dose 0 average, and percent live versus concentration and percent apoptotic versus concentration plots were constructed. Regression methods were used to predict response with the natural logarithm of the concentration. The estimated IC_{50} and associated 95% confidence interval were obtained by projection of the fitted line and pointwise confidence bounds onto the concentration axis. The IC_{50} values for each cell line after exposure to cisplatin and paclitaxel are shown below:





Cell Line	*BRCA1* and ER Status	Cisplatin IC_{50} (95% CI) µM	Paclitaxel IC_{50} (95% CI) µM
UACC-3199	*BRCA1* methylated ER negative	7.39 (4.94-10.9)	1.8 (1.1-3.8)
HCC-1937	*BRCA1* mutated ER negative	14.1 (11.6-16.4)	2.5 (1.1-4.9)
MDA-MB-231	*BRCA1* normal ER negative	21.8 (18.2-30.0)	0.13 (0.09-0.16)
MCF-7	*BRCA1* normal ER positive	> 250	> 100

As demonstrated above, the *BRCA1* methylated cell line is highly sensitive to cisplatin and resistant to paclitaxel. The methylated cells are even more sensitive than the mutated cells, likely related to minimal function of the truncated BRCA1 protein. For the *BRCA1* normal, ER negative cells, the opposite is true, demonstrating that cells with *BRCA1* promoter methylation have a unique chemosensitivity profile. These *in vitro* data support our hypothesis that *BRCA1* methylated tumors will be sensitive to DNA damaging agents and provide a strong rationale to further test this hypothesis *in vivo*. These data also raise an important concern regarding the use of paclitaxel in basal-like tumors.

Task 2: To explore the role of *BRCA1* methylation in hormone receptor negative tumors and how it might affect response to chemotherapy *in vivo*.

Secondary to poor accrual, the gemcitabine/cisplatin trial initially proposed has been closed. The trial was open for eight months, and did not accrue any patients. The main reasons for poor accrual were that gemcitabine and cisplatin are both commercially available, and many patients opted for treatment off protocol through their local oncologists. While the trial was also open to other centers that are part of the University of Chicago Phase II Network, many of the physicians reported that they also did not enroll patients to this trial as they did not feel that this regimen offered their patients a "novel" treatment. Furthermore, many community oncologists were reluctant to give cisplatin, given the length of time the treatment takes (1 hour or pre-treatment hydration and 1 hours of post-treatment hydration).

To address the factors that resulted in our inability to accrue to the previous study, another trial was designed to replace it. The goal is the same: to explore the role of *BRCA1* methylation in predicting response to DNA-damaging based chemotherapy. Given the concerns regarding cisplatin, carboplatin will be used instead. To address the concerns that the trial did not use novel agents, bevacizumab has been added. In this trial, we propose to study carboplatin and bevacizumab combination therapy in a cohort of patients with hormone receptor and HER2/*neu* negative (basal-like) metastatic breast cancer. Because these patients are likely to have BRCA1 deficiency secondary to *BRCA1* promoter methylation (and hence be more sensitive to a DNA damaging agent) and tumors with high levels of VEGF expression, we hypothesize that they are likely to respond to the combination of carboplatin and bevacizumab.

The protocol was written at the AACR/ASCO Methods in Clinical Cancer Research Workshop, and I am currently in negotiation with Genentech to supply bevacizumab. For details on rationale and trial design, see letter of intent (LOI) in appendix C.

KEY RESEARCH ACCOMPLISHMENTS

- Human breast cancer cells with *BRCA1* promoter methylation are relatively more sensitive to cisplatin and more resistant to paclitaxel, as compared to ER negative cells with normal *BRCA1* expression
- The *BRCA1* methylated cell line, UACC-3199, appears to be more sensitive to cisplatin than the *BRCA1* mutated cell line, possibly related to low level activity of the truncated BRCA1 protein in HCC-1937 cells
- The ER positive cell line, MCF-7, is relatively resistant to both cisplatin and paclitaxel
- *BRCA1* methylation confers relative resistance to paclitaxel *in vitro*, and may represent a potential mechanism of acquired paclitaxel resistance
- *BRCA1* promoter methylation occurs to some degree in a significant proportion of high-grade, hormone receptor negative breast tumors, and represents a potential therapeutic target

REPORTABLE OUTCOMES

- Based on my ability to secure research funding from the Department of Defense, I have been able to secure a faculty position at the University of Chicago (effective July 1, 2005). Based on the funding from this grant, I have been given 90% protected time to focus on developing a translational research program in breast cancer. The University has also given me a modest start up package ($100,000 for two years) to support my research while I apply for additional funding.

- The preliminary data generated from this proposal has been the basis of two Career Development Grants. I have applied for two three-year Clinical Scientist Development Grants: the ASCO Career Development Award and the Doris Duke Clinical Scientist Development Award. I will also use the preliminary data generated from this award to apply for a K23 NIH Career Development Award in October 2006.
- Abstracts for the 2005 SABCS and the 2006 AACR meetings were submitted and accepted. Both abstracts were accepted for poster presentations. For abstracts, see appendices A and B.

CONCLUSIONS

Previous studies have demonstrated that cells deficient in BRCA1 secondary to mutation are sensitive to cisplatin and resistant to paclitaxel, as compared to BRCA1 competent cells. We have demonstrated for the first time that cells deficient in BRCA1 secondary to promoter methylation are also highly sensitive to cisplatin and resistant to paclitaxel. As almost 50% of high-grade hormone receptor negative tumors have *BRCA1* promoter methylation, *BRCA1* methylation may represent both a novel therapeutic target for platinum-based chemotherapy and a mechanism for acquired resistance to paclitaxel chemotherapy.

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APPENDIX A

SABCS 2005 ABSTRACT

BRCA1 Promoter Methylation Confers Sensitivity to Cisplatin *in vitro*

Rita Nanda, MD[1], James J Dignam, PhD[2], Cindy Collins[1], Bhumi B Patel, MS[1], and Jinhua Xu, PhD[1], M Eileen Dolan, PhD[1], Olufunmilayo I Olopade, MD[1].

[1]Department of Medicine, Section of Hematology/Oncology and the [2]Department of Health Studies, University of Chicago, Chicago, IL, United States, 60637.

Background: Several groups have demonstrated that women with *BRCA1* mutations are more likely to have breast cancers that are hormone receptor and HER2/*neu* negative. Our lab has previously demonstrated that *BRCA1* promoter methylation occurs to some degree in 30% of all sporadic tumors, and up to 50% of high-grade hormone receptor negative tumors, making it much more common than germline mutation. Given the role of *BRCA1* in DNA repair, it is likely that cells deficient in BRCA1 secondary to promoter methylation will have an increased sensitivity to DNA damaging agents, as has previously been demonstrated in cells deficient in BRCA1 secondary to mutation. The role of *BRCA1* methylation in determining chemosensitivity is not yet known.

Methods: Using an *in vitro* cell line model, the relative sensitivity of *BRCA1* methylated, mutated and competent breast cancer cell lines was determined. Four breast cancer cell lines were used to determine relative sensitivity: UACC-3199 (methylated *BRCA1*), HCC-1937 (mutated *BRCA1*), MCF-7 (wildtype *BRCA1*, ER positive) and MDA-MB-231 (wildtype *BRCA1*, ER negative). Exponentially growing cells were treated with doses of cisplatin between 0.25 uM and 350 uM. Each cell line was exposed to escalating doses of cisplatin in triplicate on three separate times. Untreated cells served as a control. Cells were harvested 96 hours after drug exposure and stained with Annexin-V and DAPI. Cell survival and apoptosis were determined by flow cytometry using FACS DiVa. FlowJo FACS analysis software (version 6.1.1) was used to generate percent apoptotic and live cells. Cells that were negative for both Annexin-V and DAPI were considered live, and cells that were positive for Annexin-V and negative for DAPI were considered apoptotic. Each experiment was normalized to its own dose 0 average, and percent live vs dose and percent apoptotic vs dose curves were constructed. IC50 values were calculated from sigmoidal dose response curves.

Results: The IC50 values for the UACC-3199 and HCC-1937 cells were 16.7 uM and 78.0 uM, respectively. The IC50 values for MCF-7 and MDA-MB-231 cells were not reached, even at a dose of 350 uM. Peak percentage of apoptotic cells observed for the UACC-3199 was 40% at a cisplatin concentration of 50 uM. Peak percentage of apoptotic cells observed for the HCC-1937, MCF-7 and MDA-MB-231 cells were 20%, 16% and 21% at cisplatin concentrations of 100 uM, 350 uM, and 350 uM, respectively.

Discussion: Previous studies have demonstrated that cells deficient in *BRCA1* secondary to mutation are more sensitive to cisplatin than *BRCA1* competent cells. We have demonstrated for the first time that cells deficient in *BRCA1* secondary promoter methylation are also highly sensitive to cisplatin. As *BRCA1* methylation occurs in almost one-half of high-grade hormone receptor negative tumors, it represents a potential therapeutic target in the treatment of a subset of hormone receptor negative breast cancers. This work was supported by the US Army Department of Defense Grant W81XWH-04-1-0545.

APPENDIX B

AACR 2006 ABSTRACT

The Role of *BRCA1* Promoter Methylation in Determining Chemosensitivity *in vitro*

Rita Nanda[1], Cindy Collins[1], James J Dignam[2,], Jinhua Xu[1], M Eileen Dolan[1], and Olufunmilayo I Olopade[1].

[1]Department of Medicine and [2]Department of Health Studies, University of Chicago, Chicago, IL, United States, 60637.

Background: Several groups have demonstrated that women with *BRCA1* germline mutations are more likely to have breast cancers that are basal-like by gene expression profiling. While *BRCA1* germline mutations are uncommon, and contribute to fewer than 5% of breast cancer cases, epigenetic alterations in *BRCA1* occur with much greater frequency. Our lab has previously demonstrated that methylation of the *BRCA1* promoter occurs in almost 50% of high-grade, hormone receptor negative sporadic tumors. Given the role of *BRCA1* in both DNA repair and cell cycle regulation, it is likely that cells deficient in BRCA1 secondary to methylation will be sensitive to DNA damaging agents and resistant to microtubule inhibitors, as has previously been shown for cells deficient in BRCA1 secondary to mutation. The role of *BRCA1* methylation in determining chemosensitivity is unknown.

Methods: Using an *in vitro* model, the relative sensitivity of *BRCA1* methylated, mutated and competent cells was determined using four representative breast cancer cell lines: UACC-3199 (methylated *BRCA1*), HCC-1937 (mutated *BRCA1*), MCF-7 (wildtype *BRCA1*, ER positive) and MDA-MB-231 (wildtype *BRCA1*, ER negative). Exponentially growing cells were treated with cisplatin (CDDP) and paclitaxel. Cells were harvested 96 hours after drug exposure and stained with Annexin-V and DAPI. Cell survival was determined by flow cytometry using FACS DiVa. FlowJo FACS analysis software was used to generate percent apoptotic and live cells. IC_{50} values and 95% confidence intervals were calculated from dose response curves.

Results: The IC_{50} values and 95% confidence intervals for CDDP for the UACC-3199, HCC-1937 and MDA-MB-231 cells were 7.4 μM (4.94-10.9), 14.1 μM (11.6-16.4), and 21.8 μM (18.2-30.0), respectively. The IC_{50} value for CDDP for MCF-7 was not reached, even at a dose of 250 μM. The IC_{50} values and 95% confidence intervals for paclitaxel for the UACC-3199, HCC-1937 and MDA-MB-231 cells were 1.8 μM (1.1-3.8), 2.5 μM (1.1-4.9) and 0.13 μM (0.09-0.16), respectively. Despite dose escalation to a paclitaxel concentration of 200μM, the IC_{50} value for MCF-7 was not reached.

Discussion: Previous studies have demonstrated that cells deficient in BRCA1 secondary to mutation are sensitive to cisplatin and resistant to paclitaxel, as compared to BRCA1 competent cells. We have demonstrated for the first time that cells deficient in BRCA1 secondary to promoter methylation are also highly sensitive to cisplatin and resistant to paclitaxel. As almost 50% of high-grade hormone receptor negative tumors have *BRCA1* promoter methylation, *BRCA1* methylation may represent both a novel therapeutic target for platinum-based chemotherapy and a mechanism for acquired resistance to paclitaxel chemotherapy. This work was supported by the US Army Department of Defense Grant W81XWH-04-1-0545.

APPENDIX C

LOI FOR INVESTIGATOR INITIATED CLINICAL TRIAL

Rationale

Metastatic breast cancers that are negative for ER, PR and HER2/*neu* negative (basal-like) are aggressive and confer a poor prognosis. There is an urgent need to identify better therapies for this aggressive and difficult to treat disease.

Several groups have demonstrated that women with *BRCA1* germline mutations are more likely to have breast cancers that are basal-like by gene expression profiling[1, 2]. While *BRCA1* germline mutations are uncommon, and contribute to fewer than 5% of breast cancer cases, our lab has demonstrated that methylation of the *BRCA1* promoter occurs to some degree in almost 50% of high-grade, hormone receptor negative sporadic tumors[3]. As promoter methylation leads to transcriptional repression, we propose that such tumors will be sensitive to DNA damaging agents and resistant to microtubule inhibitors, given the role that *BRCA1* plays in both DNA repair and cell cycle. My laboratory has generated preliminary *in vitro* data that demonstrates breast cancer cells with *BRCA1* promoter methylation and low expression levels are three-fold more sensitive to cisplatin and ten times more resistant to paclitaxel, as compared to cells with normal *BRCA1* expression[8].

Hormone receptor negative tumors are highly angiogenic as measured by intratumoral microvessel density[9]. Chang and colleagues have demonstrated that the "wound response" signature, which includes genes involved in angiogenesis and matrix remodeling, is remarkably similar to the basal-like breast cancer signature, further suggesting a role for anti-angiogenic agents for these tumors[10]. VEGF is a key molecule involved in both angiogenesis and endothelial cell survival[11]. Anti-angiogenic therapy as monotherapy, however, has not been very effective in breast cancer[12]. Bevacizumab was recently shown to produce a progression free survival advantage for women with metastatic breast cancer when given in combination with paclitaxel chemotherapy as compared to those who were treated with paclitaxel alone[13].

In this trial, we propose to study carboplatin and bevacizumab combination therapy in a cohort of patients with hormone receptor and HER2/*neu* negative (basal-like) metastatic breast cancer. Because these patients are likely to have BRCA1 deficiency secondary to *BRCA1* promoter methylation (and hence be more sensitive to a DNA damaging agent) and tumors with high levels of VEGF expression, we hypothesize that they are likely to respond to the combination of carboplatin and bevacizumab.

Objectives

Primary objective: Response rate

Secondary objectives: Time to progression, correlation of response to *BRCA1* methylation

Design

Phase II, single-arm, single-center trial in patients who have had no more than one prior therapy for ER, PR and HER2/neu negative metastatic breast cancer

Population and Sample

Eligiblity Criteria:

- ER, PR and HER2/*neu* negative metastatic breast cancer
- No more than one prior chemotherapy regimen for recurrent or metastatic disease
- No prior anti-VEGF inhibitor or platinum therapy
- Measurable disease by RECIST criteria
- Tumor block must be available for correlative studies

Study Schema and Clinical Practice

	Pre-Study	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12	Off Study
Carboplatin		X			X			X			X			
Bevacizumab		X			X			X			X			
Informed consent	X													
Demographics	X													
Medical history	X													
Concurrent meds	X	X----------X												
Physical exam	X	X			X			X			X			X
Vital signs	X	X			X			X			X			X
Height	X													
Weight	X	X			X			X			X			X
Performance status	X	X			X			X			X			X
CBC, CMP	X	X			X			X			X			X
Urinanalysis	X	X			X			X			X			X
Adverse event evaluation		X----------X												X
Tumor measurements	X	Tumor measurements are repeated every 6 weeks. Documentation (radiologic) must be provided for patients removed from study for progressive disease.												X
B-HCG	X													
ER, PR and HER2/*neu* evaluation	X													
Tumor Block Collection	X													

Treatment Regimen

Carboplatin AUC 6 IV + Bevacizumab 15 mg/kg IV every 3 weeks, to be continued to disease progression or unacceptable toxicitySample Size

The primary endpoint for this phase II trial is response rate. A Simon's minimax two-stage design will be employed. The target response rate and lower bound response rate are 30% and 5% respectively. Using a significance of α=0.1 and β=0.1, the total number of patients needed is 35. In the first stage, 21 patients are treated. If 2 or more patients respond, then another 14 patients will be added, for a total of 35 patients. If less than 8 responses are observed by the end of the second stage, the regimen will be considered of low interest, and further studies involving this combination will not be planned.

Primary Evaluations

CT scans will be performed every 6 weeks. Therapy will be continued to disease progression or unacceptable toxicity. RECIST criteria will be used to assess response.

Efficacy Endpoints

Primary efficacy endpoint is response rate. The historical response rate to single agent carboplatin in the second line is 5%. The target response rate is 30%.

Safety Endpoints

There are no major safety endpoints. All adverse events will be collected and reviewed.

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